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Oxidative DNA Damage Induced by a Metabolite of 2-Naphthylamine, a Smoking-related Bladder Carcinogen

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2-Naphthylamine (2-NA), a bladder carcinogen, is contained in cigarette smoke. DNA adduct formation is thought to be a major cause of DNA damage by carcinogenic aromatic amines. We have investigated whether a metabolite of 2-NA, 2-nitroso-1-naphthol (NO-naphthol) causes oxidative DNA damage, using ^{32}P-labeled DNA fragments. We compared the mechanism of DNA damage induced by NO-naphthol with that by *N*-hydroxy-4-aminobiphenyl (4-ABP(NHOH)), a metabolite of 4-aminobiphenyl, another smoking-related bladder carcinogen. NO-naphthol caused Cu(II)-mediated DNA damage at T>C>G residues, with non-enzymatic reduction by NADH. Catalase and bathocuproine, a Cu(I)-specific chelator, inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). Some free •OH scavengers also attenuated NO-naphthol-induced DNA damage, while free •OH scavengers had no effect on the DNA damage induced by 4-ABP(NHOH). This difference suggests that the reactive species formed by NO-naphthol has more free •OH-character than that by 4-ABP(NHOH). A high-pressure liquid chromatograph equipped with an electrochemical detector showed that NO-naphthol induced 8-oxo-7,8-dihydro-2′-deoxyguanosine formation in the presence of NADH and Cu(II). The oxidative DNA damage by these amino-aromatic compounds may participate in smoking-related bladder cancer, in addition to DNA adduct formation.

Key words: Naphthylamine — DNA damage — Copper — Hydrogen peroxide

Epidemiological studies have shown that occupational exposure to 2-naphthylamine (2-NA), is strongly associated with the occurrence of bladder cancer.[1] Oral administration of 2-NA has been reported to produce bladder carcinomas in the dog and monkey, and at high dosage levels in the hamster.[1] The International Agency for Research on Cancer (IARC) has classified 2-NA as a group 1 carcinogen that is carcinogenic to humans.[1] The commercial production and usage of 2-NA have been prohibited, whereas smokers are exposed to 1–22 ng of 2-NA with every cigarette smoked.[2] There is increasing evidence that the excess of bladder cancer in smokers is attributable to aromatic amines.[3] Smoking is a risk factor for bladder cancer, in addition to lung cancer.[4–6]

DNA adduct formation is thought to be a major cause of DNA damage by these carcinogenic aromatic amines.[7–9] 2-NA is metabolically *N*-hydroxylated and glucuronidated in the liver and the *N*-glucuronide is transported to the urinary bladder. The hydrolysis of the glucuronide to *N*-hydroxynaphthylamine and subsequent DNA adduct formation are thought to be important for causing bladder cancer.[10, 11] 2-Nitroso-1-naphthol (NO-naphthol) may be produced as a metabolite of 2-NA. NO-Naphthol is formed by conversion of the *N*-hydroxy-2-naphthylamine (N-OH-NA) into nitroso compounds, followed by hydroxylation.[12, 13] Nakayama *et al.* reported that metabolites of 2-NA, such as N-OH-NA and 2-amino-1-naphthol, generated reactive oxygen species and that N-OH-NA induced DNA lesions.[14, 15]

In this study, we investigated site-specific DNA damage induced by a metabolite of 2-NA, NO-naphthol, using ^{32}P-labeled human DNA fragments from the *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene. We analyzed 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation, a marker of oxidative DNA damage, in calf thymus DNA treated with NO-naphthol. It has been reported that 8-oxodG formation can lead to DNA misreplication, resulting in mutation and cancer.[16–18]

Previously, we reported Cu(II)-mediated oxidative DNA damage induced by *N*-hydroxy-4-aminobiphenyl (4-ABP(NHOH)), which is another bladder carcinogen contained in cigarette smoke.[19] We compared the mechanism of DNA damage induced by NO-naphthol with that by 4-ABP(NHOH).

MATERIALS AND METHODS

Materials T_4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was obtained from New England Nuclear. Restriction enzymes (*Apa*I, *Ava*I, *Eco*RI, *Hin*dIII and *Pst*I) and alkaline phosphatase from calf intestine were purchased from Roche Molecular Biochemicals (Mannheim,

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Germany). NO-Naphthol was purchased from Aldrich Chem. Co. (Milwaukee, WI). 4-ABP(NHOH) was a kind gift from Dr. Mariko Tada (Aichi Shukutoku University). Copper(II) chloride dihydrate was purchased from Nacalai Tesque, Inc. (Kyoto). Diethylenetriamine-*N*,*N*,*N'*,*N''*,*N''*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were purchased from Dojin Chemicals Co. (Kumamoto). Piperidine was purchased from Wako Chemical Industries, Ltd. (Osaka). Methional (3-(methylthio)propionaldehyde) was purchased from Tokyo Kasei Co. (Tokyo). Calf thymus DNA, β-nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH), superoxide dismutase (SOD) (3000 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba).

Preparation of ^{32}P-5′-end-labeled DNA fragments obtained from the *p53* gene and the c-Ha-*ras*-1 gene DNA fragments were obtained from the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene.[20, 21] A singly ^{32}P-5′-end-labeled double-stranded 443-bp *p53* fragment (*Apa*I 14 179–*Eco*RI* 14 621) and 211-bp *p53* fragment (*Hin*dIII* 13 972–*Apa*I 14 182) were prepared from the pUC18 plasmid according to a method described previously.[22] A 337-bp c-Ha-*ras*-1 fragment (*Pst*I 2345–*Ava*I* 2681) was prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA restriction fragment.[21] The asterisk indicates ^{32}P-labeling. The 443-bp fragment was singly labeled on the 5′-end of the antisense strand of the *p53* gene and the 211-bp fragment was singly labeled on the 5′-end of the sense strand. The 337-bp fragment was singly labeled on the 5′-end of the antisense strand of the c-Ha-*ras*-1 gene.

Detection of DNA damage Detection of DNA damage and analysis of its site-specificity were performed according to a previously described method.[23] A standard reaction mixture (in a microtube; 1.5 ml) contained $CuCl_2$, NADH, NO-naphthol or 4-ABP(NHOH), the ^{32}P-labeled double-stranded DNA fragments and calf thymus DNA in 200 μl of 10 m*M* sodium phosphate buffer (pH 7.8) containing 2.5 μ*M* DTPA. After incubation at 37°C, the DNA fragments were heated at 90°C in 1 *M* piperidine for 20 min and treated as described previously.[24, 25] The treated DNA fragments were electrophoresed on an 8% polyacrylamide/8 *M* urea gel and an autoradiogram was obtained by exposing X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure using a DNA-sequencing system (LKB 2010 Macrophor).[26] A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Analysis of 8-oxodG formation by NO-naphthol Calf thymus DNA was incubated with NO-naphthol, NADH and $CuCl_2$. After ethanol precipitation, the treated DNA was digested to its component nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by a high-pressure liquid chromatograph equipped with an electrochemical detector (HPLC-ECD), as described by Kasai *et al.*[27, 28]

RESULTS

Damage to ^{32}P-labeled DNA Fig. 1 shows an autoradiogram of a DNA fragment treated with NO-naphthol in the presence of NADH and/or Cu(II). Oligonucleotides that formed after DNA cleavage were detected on the autoradiogram. In the presence of both NADH and Cu(II), NO-naphthol caused DNA damage, with 2–5 μ*M* NO-naphthol being most damaging. Piperidine treatment increased the number of oligonucleotides produced, suggesting that NO-naphthol induced not only strand breakage, but also base modification and/or liberation. NO-naphthol did not cause DNA damage in the absence of NADH or Cu(II). NO-naphthol plus NADH caused no DNA damage with Fe(II) or Fe(III) (data not shown).

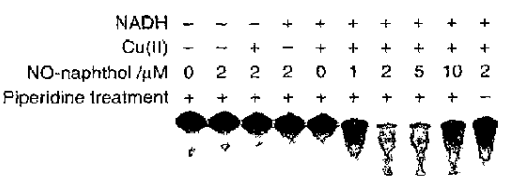

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragment incubated with NO-naphthol in the presence of Cu(II) and NADH. The reaction mixture contained ^{32}P-5′-end-labeled 337-bp DNA fragment (singly labeled double-stranded DNA), 50 μ*M*/base of calf thymus DNA, the indicated concentrations of NO-naphthol, 200 μ*M* NADH, and 20 μ*M* $CuCl_2$ in 200 μl of 10 m*M* sodium phosphate buffer (pH 7.8) containing 2.5 μ*M* diethylenetriamine-*N*,*N*,*N'*,*N''*,*N''*-pentaacetic acid (DTPA). The mixture was incubated at 37°C for 2 h, followed by a piperidine treatment, as described in "Materials and Methods." After denaturation, the DNA fragments were electrophoresed on an 8% polyacrylamide/8 *M* urea gel and the autoradiogram was obtained by exposing X-ray film to the gel.

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Effects of scavengers and bathocuproine on DNA damage induced by NO-naphthol or by 4-ABP(NHOH) in the presence of NADH and Cu(II) Some typical free •OH scavengers, dimethylsulfoxide (DMSO) (data not shown) and ethanol efficiently inhibited the DNA damage induced by NO-naphthol, as shown in Fig. 2A, while none of the typical free •OH scavengers had any effect on the DNA damage induced by 4-ABP(NHOH), as shown in Fig. 2B. In Fig. 2A, another •OH scavenger, mannitol partially attenuated the NO-naphthol-induced DNA damage, whereas sodium formate did not. Bathocuproine, a Cu(I)-specific chelator, catalase and methional inhibited DNA damage induced by NO-naphthol and by 4-ABP(NHOH). SOD also attenuated NO-naphthol-induced DNA damage, but did not affect 4-ABP(NHOH)-induced DNA damage.

Comparison of site-specific DNA damage by NO-naphthol and by 4-ABP(NHOH) in the presence of NADH and Cu(II) An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensities of DNA cleavage products from the human *p53* tumor suppressor gene (Fig. 3) and from the c-Ha-*ras*-1 protooncogene (Fig. 4). NO-naphthol was observed to induce piperidine-labile sites preferentially at T>C>G residues in the presence of NADH and Cu(II). 4-ABP(NHOH) was observed to induce piperidine-labile sites specifically at thymine residues in the presence of NADH and Cu(II) (Figs. 3B and 4B). NO-naphthol induced piperidine-labile sites at cytosine and guanine residues, in addition to thymine residues similar to those frequently damaged by 4-ABP(NHOH). We also detected guanine damage induced by NO-naphthol and by 4-ABP(NHOH) as 8-oxodG, which is a piperidine-inert base modification. 8-OxodG formation should occur adjacent to piperidine-labile T sites, since double base lesions at 5′-TG-3′ or at 5′-GT-3′ were previously reported in oxidative DNA base damage.[29,30]

In the cases of both NO-naphthol and 4-ABP(NHOH), the ACG sequence complementary to codon 273 of the *p53* gene was significantly damaged. Codon 273 of the *p53* gene is a well-known hotspot of lung cancer, and it is also reported to be a mutational hotspot of bladder cancer in relation to smoking.[31]

Formation of 8-oxodG in calf thymus DNA Using HPLC-ECD, we measured the 8-oxodG content of calf thymus DNA treated with NO-naphthol in the presence of

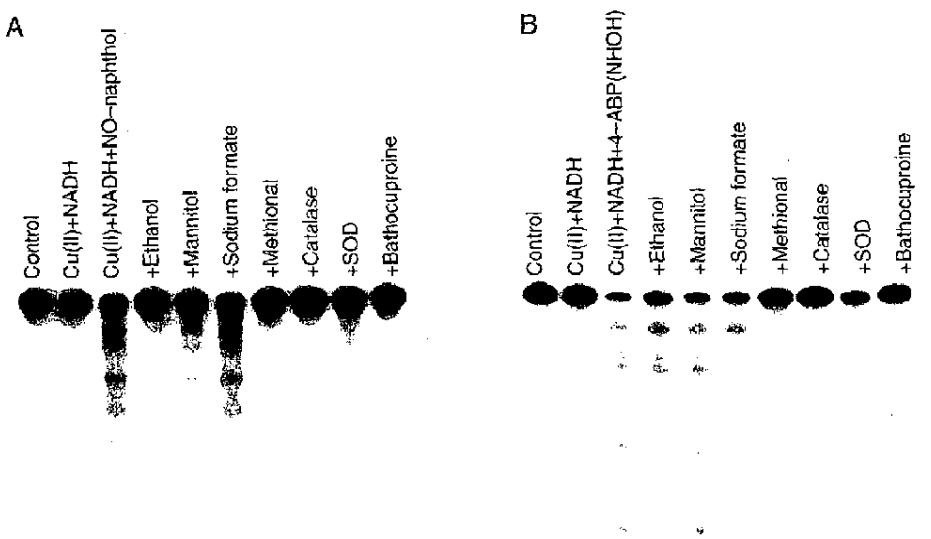

Fig. 2. Effects of scavengers and bathocuproine on DNA damage induced by NO-naphthol in the presence of Cu(II) and NADH. The reaction mixture contained ^{32}P-5′-end-labeled 443-bp (A) or 211-bp (B) DNA fragment, 50 μM/base of calf thymus DNA, 2 μM NO-naphthol (A) or 0.5 μM 4-ABP(NHOH) (B), 200 μM NADH and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37°C for 1 h, followed by piperidine treatment. The DNA fragment was analyzed as described in the legend to Fig. 1. The concentrations of scavengers and bathocuproine were as follows: 5% (v/v) ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.1 M methional, 30 units of catalase, 30 units of SOD, 50 μM bathocuproine.

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NADH and Cu(II) (Fig. 5). NO-naphthol at 2–5 μM most effectively caused 8-oxodG formation on native DNA. The amount of 8-oxodG increased with the concentration of NADH. In the absence of NADH, NO-naphthol and Cu(II) together did not stimulate 8-oxodG formation above control levels (data not shown).

DISCUSSION

The present study showed that NO-naphthol, a metabolite of 2-NA caused oxidative DNA damage including 8-oxodG in the presence of NADH and Cu(II). NO-naphthol plus Cu(II) did not cause DNA damage in the absence of NADH, indicating that the reduction of NO-naphthol by NADH was essential. To clarify the kinds of reactive species at relatively low concentrations of NO-naphthol, we examined the effects of scavengers on the DNA damage induced by NO-naphthol. Both catalase and bathocuproine inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). The formation of 8-oxodG by 2–5 μM NO-naphthol was more efficient in double-stranded DNA than in single-stranded DNA (data not shown). This sug-

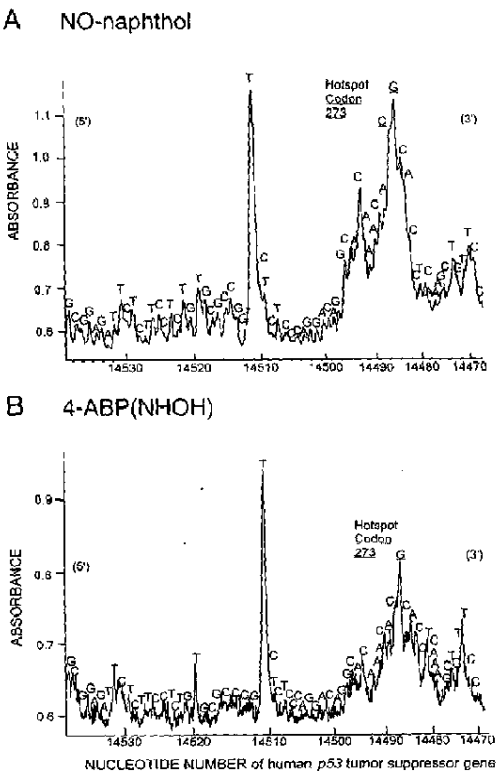

Fig. 3. Comparison of site-specific DNA damage by NO-naphthol and 4-ABP(NHOH). The reaction mixture contained ^{32}P-5′-end-labeled 443-bp fragment, calf thymus DNA [(A) 50 μM, (B) 5 μM], 2 μM NO-naphthol (A) or 1 μM 4-ABP(NHOH) (B), 200 μM NADH and 20 μM $CuCl_2$, in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The 443-bp fragment was singly labeled on the 5′-end of the antisense strand of the human *p53* tumor suppressor gene. The reaction mixture was incubated at 37°C for 1 h (A) or 30 min (B). After piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. The horizontal axis shows the nucleotide number. (A) NO-naphthol. (B) 4-ABP(NHOH).

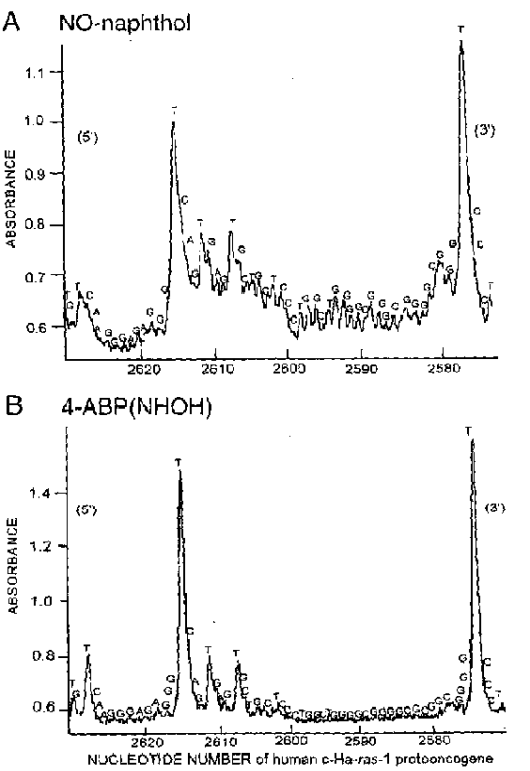

Fig. 4. Comparison of site-specific DNA damage by NO-naphthol and 4-ABP(NHOH). The reaction mixture contained ^{32}P-5′-end-labeled 337-bp fragment, calf thymus DNA [(A) 50 μM, (B) 5 μM], 2 μM NO-naphthol (A) or 1 μM 4-ABP(NHOH) (B), 200 μM NADH and 20 μM $CuCl_2$, in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The 337-bp fragment was singly labeled on the 5′-end of the antisense strand of the human c-Ha-*ras*-1 protooncogene. The reaction mixture was incubated at 37°C for 1 h (A) or 30 min (B). After piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. The horizontal axis shows the nucleotide number. (A) NO-naphthol. (B) 4-ABP(NHOH).

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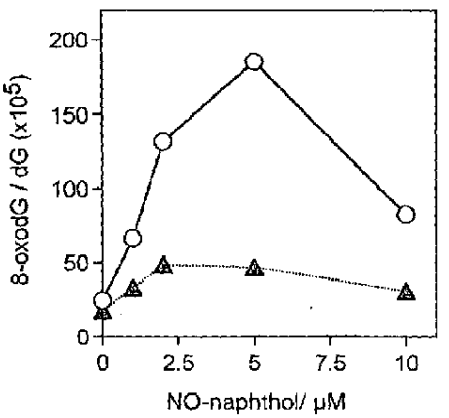

Fig. 5. Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by NO-naphthol in the presence of NADH. Calf thymus DNA (50 μM/base) was incubated with the indicated concentrations of NO-naphthol and 20 μM $CuCl_2$ in the presence of various concentrations of NADH for 1 h at 37°C. After ethanol precipitation, DNA was enzymatically digested into nucleosides, and 8-oxodG formation was measured with an HPLC-ECD as described in "Materials and Methods." ○ NADH 200 μM, ▲ NADH 100 μM.

gests that double-stranded structure is important for DNA damage. At relatively high concentrations of NO-naphthol, the amount of DNA damage decreased, suggesting that two molecules of NO-naphthol may bischelate with one molecule of copper to generate an inactive form. This is supported by the report that NO-naphthol chelates metal ions, such as iron, copper and zinc.[32]

Based on these results and references, we propose the following possible mechanism (Fig. 6). 2-NA is metabolized to NO-naphthol through several metabolic steps.[10, 12, 13] NO-naphthol is non-enzymatically reduced to hydronitroxide radical by NADH. This is supported by an electron-spin resonance (ESR) study that has confirmed the existence of the radical.[33, 34] We previously reported that aromatic nitroso compounds can be easily reduced to hydronitroxide radicals by NADH, forming a redox cycle.[19, 35, 36] Auto-oxidation of hydronitroxide radical to the nitroso compound (NO-naphthol) occurs coupled with generation of $O_2^{\cdot-}$, and $O_2^{\cdot-}$ mediated reduction of Cu(II) to Cu(I). Then auto-oxidation of Cu(I) to Cu(II) occurs coupled with generation of $O_2^{\cdot-}$, which is dismutated to H_2O_2. When iron was used instead of copper, NO-naphthol with NADH did not cause DNA damage in this system, though it remains possible that iron ions mediate DNA

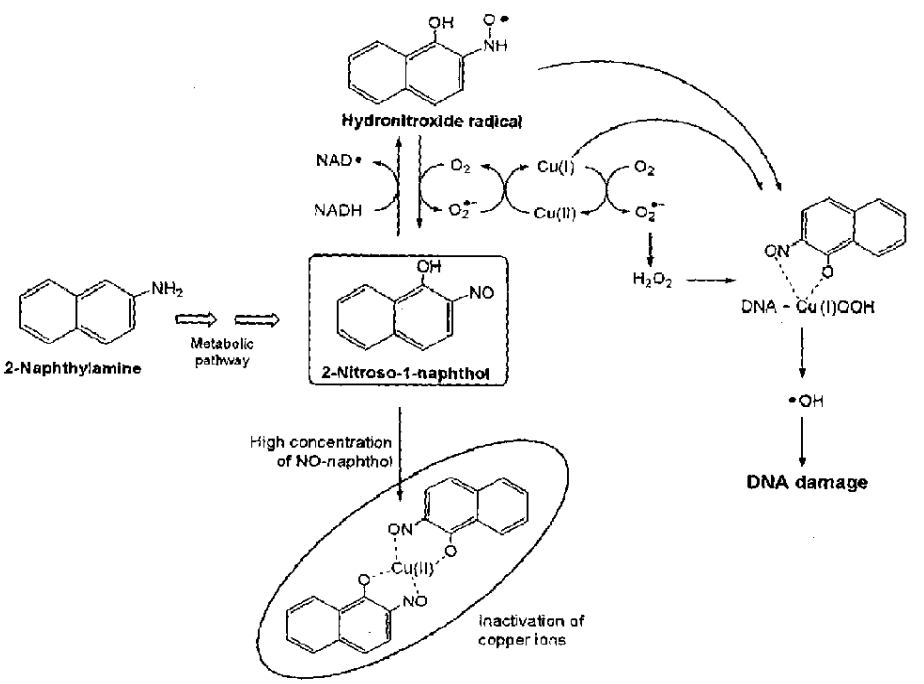

Fig. 6. A proposed mechanism for oxidative DNA damage induced by a 2-NA metabolite in the presence of Cu(II) and NADH.

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damage by NO-naphthol *in vivo*. In the case of iron, even if Fe(III) is reduced to Fe(II) by the formed $O_2^{\cdot-}$, the reaction of Fe(II) with O_2 is much slower than that of Cu(I).[37] This could be one of the reasons why iron does not work in this system.

Typical •OH scavengers partially inhibited DNA damage induced by NO-naphthol, while they showed no inhibitory effect on DNA damage induced by 4-ABP(NHOH). Generally, it is known that copper exists in the nucleus and is closely associated with chromosomes and DNA bases *in vivo*.[38] H_2O_2 interacts with Cu(I) to form DNA-copper-hydroperoxo complexes, causing DNA damage. The non-inhibitory effect of typical free •OH scavengers observed in the case of 4-ABP(NHOH), can be explained by assuming that DNA damage is induced by •OH generated in very close proximity to the DNA by the bound metal ion. On the other hand, NO-naphthol which intercalated in DNA, may react with the bound metal ion via its two functional groups and keep the metal ion relatively far from DNA. The DNA base damage by 4-ABP(NHOH) was more specific than that by NO-naphthol. It has been reported that free •OH caused DNA damage at any nucleotide, showing little site specificity.[24, 39] Therefore, it can reasonably be considered that the reactive species formed by NO-naphthol has a free •OH-like character to a greater extent than in the case of 4-ABP(NHOH).

Carcinogenicity of amino-aromatic compounds has been explained in terms of DNA-adduct formation.[7–9] The DNA adducts derived from NA, formed by covalent binding through the activated nitrogen, have been identified.[11] On the other hand, Nagao *et al.* pointed out that there was no direct correlation between DNA-adduct levels and cancer incidences induced by heterocyclic amines.[40] In addition, free radicals and subsequently formed reactive oxygen species may participate in aromatic amine carcinogenesis.[14, 41] It has been reported that the levels of 8-oxodG and its repair activity are increased with cigarette smoking,[42–44] and that 8-oxodG levels were increased in tissues of lung cancer patients.[45] Smoking is a risk factor for bladder cancer as well as lung cancer.[3, 4] The present study has demonstrated that a metabolite of 2-NA induces oxidative DNA damage, as does the 4-ABP metabolite. NO-naphthol is likely to be involved in bladder carcinogenesis through metabolite formation around the bladder, although the genotoxicity of NO-naphthol is probably not specific to urothelial cells. The oxidative DNA damage by these amino-aromatic compounds may participate in smoking-related bladder cancer, in addition to DNA adduct formation.

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